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Antimicrobial Peptide Coatings for Hydroxyapatite: Electrostatic and Covalent attachment of
Antimicrobial Peptides to Surfaces.

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Abstract

The interface between implanted devices and their host tissue is complex and is often optimised for maximal integration and cell adhesion. However, this also gives a surface suitable for bacterial colonisation. We have developed a novel method of modifying the surface at the material-tissue interface with an antimicrobial peptide coating to allow cell attachment while inhibiting bacterial colonisation. The technology reported here is a dual antimicrobial peptide coating. The dual coating consists of antimicrobial peptides covalently bonded to the hydroxyapatite surface, followed by deposition of electrostatically bound antimicrobial peptides. The dual approach gives an efficacious coating which is stable for over 12 months and can prevent colonisation of the surface by both gram positive and gram negative bacteria.

Background

Infection is recognised as one of the leading complications of bone replacement implant operations within the orthopaedics fields, with 2.8 % of operations contracting a surgical site infection¹. While acute infections occur immediately, it is common for some infections, particularly prosthetic joint infections, to be detected over 1 year following the implantation

of the device. These infections are often a complex and challenging complication to prevent or treat².

The bacteria most commonly associated with infection in orthopaedic implants are Gram positive bacteria - coagulase-negative *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus aureus* (*S. aureus*), (both methicillin-sensitive and resistant) and *Enterococcus spp.*, which are responsible for 75 % of all implant infections³ and Gram negative bacteria - *Pseudomonas aeruginosa* (*P. aeruginosa*). Current clinical practice for treating infection around an implant site is an extended course of antibiotics. However, systemic and oral administration of antibiotics often results in low availability at the site of infection⁴. Bacteria can also colonise the implant forming a biofilm on the surface resulting in infection⁵. This type of infection is difficult to treat as the dense extracellular matrix associated with the biofilm acts a barrier against detergents and antibiotics, with further complications presented by the rise of multi-resistant bacteria such as methicillin-resistant *S. aureus* (MRSA)⁶. Well established infection around the implant results in necrosis of the surrounding tissue often requiring surgical debridement. Extensive tissue necrosis breaks down the bone-implant interface, causing loosening of the implant and loss of limb function, which then requires surgical retrieval and replacement of the implant^{7,8}. Implant retrieval not only incurs significant financial costs and patient discomfort, but the procedure also results in further bone tissue loss, meaning that implant replacement can only be performed a limited number of times over the lifetime of the patient.

The use of titanium for the manufacture of bone replacement implants is well established as a result of its high strength and stiffness properties, along with its corrosion resistance and excellent biocompatibility in bone replacement applications⁹. Hydroxyapatite (HA) is a mineral with similar chemical composition to the mineral fraction of human bone and is

used as a coating on metal implants to promote osseointegration^{10,11}. Currently, HA coatings are being used to coat cement-less implants and to promote bone ingrowth¹². Many reports suggest that HA coatings on metal implants precipitate faster than bone fixation, resulting in a reduction of pain and recovery time for implant patients^{13,14}. Self-cleaning surfaces are well established in the natural world to prevent bacterial adhesion¹⁵.

There are an array of technologies which can be used to deposit coatings on surfaces. These include the deposition of patterned hydroxyapatite^{16,17}, inorganic surfaces binding peptides¹⁸ and the incorporation of silver nanoparticles¹⁹. Numerous strategies for incorporating antimicrobial agents with orthopaedic implants have been investigated, including: i) coating titanium with polymers loaded with antibiotics for sustained release^{20,21}, ii) deposition of antimicrobial ion coatings such as Ag or Cu^{22,23} and, iii) altering the topography of the surface²⁴.

These approaches have also been exploited for incorporating antimicrobials into HA (and other calcium phosphate-based) coatings. Silver ions have been shown to display high antimicrobial efficiency and are easily incorporated into materials such as hydroxyapatite through an ion exchange process^{25,26,27,28}. Antimicrobial agents have been added on to the implant surface using dip/spray coating, which rely on adsorption of the agent on to the implant surface. However, adsorbed molecules are often weakly bound to the implant surface and can be rapidly desorbed once under physiological conditions. The leaching of the antimicrobial from the surface into the surrounding area following implantation may provide relatively short term potency in the local tissue area¹⁹. However, rapid desorption of the antimicrobial correspondingly reduces the antimicrobial potency at the implant surface. Studies on titanium have shown that it is possible to utilise the strong association between the titanium oxide passivation layer and catechol chemistry to give a stable binding of an

antibiotic to the surface²⁹. However, this chemistry is not directly translatable to hydroxyapatite. Also the inclusion of antibiotics such as cefotaxime is not desirable in a clinical setting as cefotaxime-resistant species are already established³⁰. If an infection occurs from these bacteria the coating would then be ineffectual.

Antimicrobial peptides (AMPs) have been identified as promising active agents against bacterial and fungal microorganisms due to their broad spectrum of activity coupled with low toxicity. However, clinical applications and commercial development of these compounds is still very limited³¹. Difficulties in their production, poor optimisation of their properties and efficacy, together with high manufacturing costs have contributed to the slow transfer from research to clinical practice and development of commercial products³².

The rise of antibiotic-resistant bacteria is stimulating significant interest in applying AMPs to biomedical devices and translating them to clinical use. The main sectors of application of AMPs are mainly those of prevention and control of infections in humans and animals, while other opportunities can also be foreseen such as food preservation systems and utilization in agricultural and environmental protection.

Antimicrobial coatings have many uses in medicine and include coatings for implants³³, catheters³⁴, and dressings for wounds³⁵. There are several AMP compounds that are undergoing clinical trials to consider combined antimicrobial and immune-modulatory functions. However, at present no drugs based on AMPs have been approved³⁶. Due to their chemical nature (peptides), oral and intravenous administration of AMPs poses problems due to possible reduction or neutralization of the active ingredient or induction of an allergic reaction³⁷. On the other hand, AMP derived drugs appear very promising compounds for topical formulations, for example for the treatment of skin burns/lesions, open wounds, and the protection of implanted devices ranging from catheters to contact

lenses, stents and artificial tissue substitute applications. Natural methods of combating infection in the body include defence proteins such as Defensins. Defensins are small proteins purified from granulocytes that display high levels of antimicrobial activity³⁸. Defensins counteract bacterial invasion of the body through direct and indirect pathways. The direct pathway involves attacking the invading pathogen by inducing pore formation in the membrane of the cell, leading to cell lysis³⁹. Novel synthetic peptides based on plant defensins have been designed and shown to exhibit good levels of antimicrobial activity⁴⁰. Scudiero *et al* used human β defensins as a model for novel synthetic peptides and demonstrated good antimicrobial activity. However, this activity was reliant on the localised ion concentration although the synthetic analogues were not as susceptible to this effect as the natural defensin⁴¹.

In this work we demonstrate an alternative method for imparting antimicrobial activity upon orthopaedic implants. The method employs two mechanisms in tandem for binding a defensin based antimicrobial peptide (AMP) to a hydroxyapatite surface with the aim of providing both a robust long-term antimicrobial coating of the implant and facilitating partial release of the antimicrobial payload into the surrounding tissue. This uses two different methodologies, a covalent attachment of the peptide (cAMP) which gives a 'permanent' coating and an electrostatic attachment of the peptide (eAMP) which is released from this short term surface coating into the area surrounding the implant sterilising the tissue around the implant. Employing both coatings simultaneously on the same surface gives a dual coating (dAMP) which has allowed us to create a multi-functional antimicrobial surface, which can sterilise the surgical site and prevent colonisation of the implant for an extended period of time.

Materials and methods

Materials

All reagents were purchased from Sigma (Poole, UK), unless otherwise stated. Peptides were purchased from Genscript (Piscataway, USA). Bacteria were obtained as patient isolates from the Queen Elizabeth Hospital, Birmingham, UK.

Hydroxyapatite synthesis

HA (for both electrostatic and covalent studies) was prepared using the following steps. Aqueous solutions of calcium nitrate 1 M (Fisher Scientific UK Ltd., Loughborough, UK) and diammonium hydrogen phosphate dibasic 0.6 M were prepared and the pH adjusted to 10 through drop wise addition of 37-39% ammonium hydroxide. The phosphate salt solution was added drop wise to the calcium salt solution using a burette while stirring and maintaining a pH of 10 through further addition of ammonium hydroxide. After ageing overnight under stirring, the nanocrystals were washed by five cycles of centrifugation at 4000 rpm and resuspension with deionised water before filtering. Typical yield of solid product was approximately 30 g, with 1 g resuspended at 1 mg per mL in deionised water adjusted to pH 7 and dried overnight in an oven at 60 °C to give hydroxyapatite as a powder. HA pellets for electrostatic studies were prepared using dried hydroxyapatite powder mixed with deionised water (830 μ L for each gram of HA) to form a thick paste. The paste was put in a homemade PTFE mould and incubated at 60 °C overnight before sintering at 1100 °C for 6 hours with a temperature ramp rate of 5 °C/min.

Thiol functionalised hydroxyapatite (tHA) was prepared for covalent hydroxyapatite studies.

The thiol functionalisation solution consisted of 3 % (v/v) (3-mercaptopropyl) trimethoxysilane (MPTS) stock solution, 3 % (v/v) ultra-pure deionised water and 94 % (v/v) methanol. The water-methanol mixture was prepared before adding the MPTS. The pH was adjusted slowly to 4 by addition of 1 M hydrochloric acid and left to hydrolyse for 4 hours.

The hydrolysed MPTS solution was then pH adjusted to 7 by addition of ammonium hydroxide before adding the cut HA pellets and leaving it to react overnight while on a flat shaker plate running at 300 rpm (ThermoScientific Inc., Waltham, MA, USA).

No more than 30 pellets were placed in each reaction bottle at a time in order to ensure adequate separation and coating of the pellets. The pellets were carefully washed five times in 20 % methanol before drying in an oven for 2 hours at 60 °C.

eAMP coating of HA

The moulded HA pellets which had been prepared as described above were incubated in eAMP (5(6)-Carboxyfluorescein) – RRRRRRGALAGRRRRRRGALAGEEEEEEE, (0.9 mM) in phosphate buffered saline (PBS) for 30 minutes. The coated pellets were then removed from the solution and washed five times with PBS and stored at room temperature before being used for studies.

cAMP coating of hydroxyapatite

tHA pellets which had been prepared as described above were incubated in N,N-dimethylformamide (DMF) (10 mL), succinimidyltrans-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC) (0.2 M) and the cAMP (5(6)carboxyfluorescein) RRRRRRGALAGRRRRRRGALAG (0.9 mM) for 12 hours with shaking. The covalently coated

pellets were removed from the solution and washed five times with DMF and five times with PBS before being used in studies.

dAMP coating of thiol-hydroxyapatite

tHA pellets which had been prepared as described above were incubated in DMF (10 mL), SMCC (0.2 M) and the cAMP 5(6)carboxyfluorescein) RRRRRRGALAGRRRRRRGALAG (0.9 mM) for 12 hours with shaking. The covalently coated pellets were removed from the solution and washed five times with DMF and five times with PBS. The pellets were then incubated in eAMP (5(6)-Carboxyfluorescein) – RRRRRRGALAGRRRRRRGALAGEEEEEEE, (0.9 mM) in phosphate buffered saline (PBS) for 30 minutes. The coated pellets were then removed from the solution and washed five times with PBS and stored at room temperature before being used for studies. Control pellets were uncoated HA which had been washed in DMF (5 x1 mL) and PBS (5 x 1 mL).

AMP coating of hydroxyapatite

As a control for release studies HA pellets were coated with RRRRRRGALAGRRRRR 5(6)carboxyfluorescein) RRRRRRGALAGRRRRRRGALAG. The peptide was added to PBS (10 mL). HA pellet was added to the solution and incubated for 12 hours with shaking. The pellets were removed from solution and washed five times with PBS.

Quantification of peptide on hydroxyapatite

Pellets were coated as described above. The amount of peptide remaining in the reaction vessel and the washes was quantified using fluorescence of the 5(6) carboxyfluorescein tag.

This was then used to calculate the remaining peptide which had been immobilised on the surface.

Release of AMP from eAMP and cAMP surfaces

eAMP, cAMP and AMP coated pellets were incubated in PBS (1 mL) at 37 °C and the solution was changed at each time point. The solutions were stored in the dark at 4 °C and the fluorescence of the 5(6) carboxyfluorescein tag was monitored at excitation/emission 495/525 nm on a Wallac 1420 plate reader (Perkin Elmer, London, UK). Percentage values were calculated from the known concentration immobilised on the surface.

Stability of dAMP coatings

HA pellets were coated as described above. Twelve pellets were split into 4 treatment groups of 3 samples: 1) HA, 2) eAMP-HA, 3) tHA, 4) cAMP-tHA. The surface of the pellets were imaged immediately following the coating procedure to ensure the coating was present. The pellets were then incubated for 12 months at room temperature (20-24 °C) in simulated body fluid prepared from literature sources⁴².

At 7, 14 day 1 month and 12 month time points the pellets were removed from the simulated body fluid solution, dried and viewed with an Axioplan-2 fluorescence microscope and images of their surfaces obtained with Axiovision software (both Carl Zeiss Ltd, Hertfordshire, UK) to detect residual fluorescent dAMP. Peptide on the surface was quantified using established methods⁴³. Briefly, each of the pellets was imaged on 9 different areas of the surface (total 27 images/treatment). The levels of the peptide on the surface were quantified using Image J (National Institutes of Health). All images were taken

at the same exposure, the % of fluorescent pixels above a standardized background threshold calculated using Image J.

Proteolytic stability

cAMP coated pellets were coated as described and the levels of fluorescence on the surfaces quantified. The pellets were incubated in trypsin-edta (1 mL, 0.02 M) or human leukocyte elastase (1 mL, 0.6 µg/mL Merck Millipore, Nottingham, UK) for 2 hours. The pellets were removed from the solution and the surfaces washed three times in PBS (1 mL). The pellets were air dried in the dark and imaged and quantified as previously described.

Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray

Spectroscopy (EDX) of surfaces

Surfaces were prepared as previously described for fluorescence microscopy. The samples were studied on a Philips XL30 FEG scanning electron microscope. Imaging was carried out in triplicate and images shown are representative of each surface. After SEM samples were submitted for EDX. Samples were run in triplicate and spectra sites were chosen at random across each surface. Data presented is the averages of all spectra.

Antimicrobial efficacy testing of AMP on laboratory strains of bacteria

The minimum inhibitory concentration (MIC) of the prepared AMP against various bacteria was determined by the broth dilution method according to the recommendations of the British Society of Antimicrobial Chemotherapy⁴⁴. Briefly, serial dilutions of peptide solutions were prepared at 3.7 – 0.002 mM concentrations made up in lysogeny (LB) broth and tested

against *S. aureus* and *S. epidermidis*, the lowest concentration required to inhibit bacterial growth was used.

Antimicrobial resistance studies

Bacterial resistance studies were then carried out by incubating each bacteria in LB broth supplemented with 50 % of the concentration of the peptide required to get an MIC concentration. Incubation was carried out at 37 °C for 12 hours and the MIC test repeated on the pre-treated bacteria as described above.

Further resistance studies were carried out by incubating each bacteria with 0.003 mM AMP concentration and then doubling the concentration of AMP every 24 hours until half the MIC value was reached. At each time point, bacteria were sampled using the MIC assay and the MIC noted. These studies allow one to establish how easily resistance might be developed to the AMP peptide.

Antimicrobial tests of AMP surfaces

Antimicrobial coated HA surfaces were tested by incubating cAMP-tHA/dAMP-tHA and uncoated pellets were inoculated with 10 µl of $\sim 10^9$ colony forming units (cfu)/ml of coagulase-negative *S. epidermidis*, *S. aureus* and *P. aeruginosa*. Pellets were incubated for 24 hours at 37 °C. The optical density of LB Broth around the pellet was measured at 600 nm using a spectrophotometer (Jenway, Stone, UK). The pellet surface was then swabbed and plated out on LB Agar. The plates were incubated at 37 °C for 24 hours and the number of visible colonies on the plate counted.

Effect of serum incubation on antimicrobial efficacy

dAMP pellets were prepared as previously described. The pellets were incubated in fetal calf serum (1mL) for 4 hours. The pellets were washed with PBS and then the antimicrobial efficacy was tested against *S. aureus* as described above.

Long Term Antimicrobial Studies

HA and dAMP-tHA coated pellets were incubated in LB Broth (1 mL) and inoculated with 10 μ l of $\sim 10^9$ colony forming units (cfu)/ml of coagulase-negative *S. epidermidis*. At 7, 14 and 21 days the broth was tested for optical density to determine bacterial number and the surfaces swabbed and plated out on LB Agar. The agar plates were incubated at 37 °C overnight and the colonies counted.

Toxicity studies of AMP and dAMP with mammalian cells

Toxicity studies were carried out using osteoblast precursor cells (MC3T3). Cells were detached from a confluent flask and seeded at 50,000 cells/well. The media (DMEM, fetal bovine serum (10 % v/v), penicillin/streptomycin (1 % v/v) was supplemented with 37 μ g of peptide/well. At each time point the cells were detached from the plate using trypsin (200 μ l). The cells were counted using a haemocytometer.

dAMP-tHA were placed in 12 well plate and seeded with 50,000 cells and covered with media (1 mL). Samples were prepared separately for resazurin and cell counts. Samples for resazurin had media removed and were washed with PBS. The pellets were resuspended in fresh media (1 mL) with resazurin (20 μ l, 15mg/mL) and incubated for 2 hours at 37 °C.

Aliquots (200 μ l) of the media were removed and added to a 96 well plate. The absorbance was read at 570 nm on a (Wallac Victor 3 1420 multilabel counter). Samples for cell number

had the media removed and the pellet incubated in trypsin (200 µl) for 5 minutes at 37 °C. The cell suspension was added to a haemocytometer and counted.

Toxicity studies were carried out using SAOS-2 cells (Osteosarcoma cells, ECACC, used at passages 10-14).

Cells were detached from a confluent flask and seeded at 5,000 cells/well. The media (DMEM, fetal bovine serum (10 % v/v), penicillin/streptomycin (1 % v/v) was supplemented with 37 µg of peptide/well. At each time point the cells were detached from the plate using trypsin (200 µl). The cells were counted using a haemocytometer.

Electrostatic and covalently coated HA pellets were prepared as previously described. SAOS 2 cells were seeded onto dAMP coated and uncoated HA pellets at a density of 5000 cells/pellet in 500 µl of McCoy's 5a media supplemented with 10% FBS and 1 % Penicillin/Streptomycin and the media changed every 3-4 days. At each sample time point the HA pellets with their attached SAOS-2 cells were removed from the media and gently washed with PBS (3 x 1 mL). The cells growing on the HA pellets were then tested for metabolic activity using alamar Blue following established protocols³⁵. Briefly, alamar blue dye (10 µl) was added to the media surrounding the cell coated pellets and incubated for 2 hours and then the absorbance of the media was read at 570 nm on a Glomax multi detection system (Promega, Southampton, UK). The cell bearing HA pellets were then washed in PBS to remove any dead cells, then placed in sterile water and frozen at – 80 °C. The samples were subjected to a three cycle freeze thaw process to lyse the cells and release their DNA into solution, and the DNA content of each sample was measured using Hoechst 33342 dye to assess SAOS-2 cell survival⁴⁵.

Statistics

All statistical analyses were carried out using SPSS 17.0 (IBM SPSS Inc., Chicago, IL) and data were presented as mean \pm SEM. The Shapiro-Wilk test was used to ensure all data was normally distributed before parametric testing using a one-way ANOVA with Tukey *post-hoc* test or a Mann-Whitney U-Test. The statistical significance threshold was $p < 0.05$.

Results

Peptide design and synthesis

AMPs were designed using sequence matching from human α and β defensins. Amino acid residue properties including positive/negative charges and hydrophilic/hydrophobic patterns were compared across the different defensins and condensed into a small peptide sequence pattern which was then synthesised using solid phase peptide synthesis (Figure 1). All peptides were additionally tagged with 5(6) carboxyfluorescein (5(6)-CF) to allow visualisation of the peptide on attachment surfaces and peptide quantification. The individual peptide sequences generated, provided a toolkit of activity (antimicrobial activity, surface binding activity or both antimicrobial and surface binding activity) which allowed a mix and match approach to building candidate peptides for binding to specific surfaces (e.g. the RRRRRRGALAGRRRRRRGALAGGGGEEEEEEEE peptide sequence gave a broad spectrum high activity AMP which binds to HA electrostatically).

The cAMP coating was characterised using energy-dispersive X-ray spectroscopy (EDX) (Table 1). This data showed that there is a significant increase in the presence of Si between HA (0 ± 0) and tHA (0.2 ± 0.08), ($p = 0.002$). This was mirrored by the increase in sulfur with HA (0 ± 0), tHA (0.37 ± 0.10), ($p = 0.000$). The increase in sulfur can be attributed to

the sulfhydryl groups of MPTS, demonstrating thiol modification of the surface. This was further supported by raman spectroscopy (Supplementary Information) which showed a peak at 2572 cm⁻¹, which corresponds to the S-H stretching mode, as well as 2890 and 2923 cm⁻¹ which are the CH₂ vibrations of the propyl chain of the MPTS. The cAMP-tHA was characterised by EDX. The spectra showed a significant increase in the amount of carbon present on the surface between tHA (8.78 ± 0.44) and the cAMP-tHA (25.95 ± 4.76), (p<0.000) (Table 1). The increase in carbon is consistent with the incorporation of the peptide onto the surface. The amount of peptide incorporated onto the surface was determined to be 0.54 mol/cm² (cAMP) and 1.78 mol/cm² (eAMP). The calculations also showed a 1:1.2 cAMP to eAMP ratio in dAMP coated pellets.

Raman spectroscopy was also used to examine the composition of the hydroxyapatite to determine if tricalcium phosphate (TCP) was present (supplementary Information). The spectra showed hydroxyapatite peaks with slight peak broadening suggesting a small amount of TCP was present in the pellets.

Surfaces with either eAMP or cAMP coating were imaged for surface coverage using fluorescence microscopy (Figure 2a-f), which showed that AMP was successfully bound to the surface with good coverage immediately following both covalent and electrostatic attachment. The coated HA pellets were incubated in simulated body fluid for 1 month with regular changes of fluid before fluorescent reimaging to visualise residual AMP (both eAMP and cAMP), and this showed that at this time point all of the electrostatically bound peptide was removed from the HA surface (Figure 2a-c). However, with covalent functionalisation

there was still coverage of the cAMP on the tHA surface following the incubation period (Figure 2d-f).

Incorporation of the binding sequences into the AMP structure allowed the electrostatic binding to HA surfaces to give a sustained peptide release profile over 24 hours. The release profile showed that 100 % of the eAMP was released over 96 hours (Figure 2 i). This is significantly higher than cAMP samples which showed no release in the same time period. While the AMP coating with no specific attachment method to the HA showed 100 % release at 8 hours. The long term stability of the coating was examined after incubation in PBS for 12 months (Figure 2g). This showed no significant differences in the levels of fluorescence immediately after coating ($1.3 \times 10^6 \pm 7.2 \times 10^4$) compared to after incubation for 12 months ($1.2 \times 10^6 \pm 3.8 \times 10^4$). The surfaces also showed to be resistant to proteolysis from both human leukocyte elastase ($1.3 \times 10^6 \pm 2.3 \times 10^4$) and trypsin ($1.3 \times 10^6 \pm 5.6 \times 10^4$) (Figure 2h). All the samples were significantly higher than uncoated samples (83 ± 85).

The surfaces were also characterised by SEM (Figure 2a-c). This showed that there were no differences in HA structure between HA, tHA and dAMP-tHA. The images show crystalline structures consistent with hydroxyapatite.

Antimicrobial efficiency of antimicrobial peptide sequences in solution

AMP sequence (RRRRRRGALAGRRRRRRGALAG) was tested in solution against a broad spectrum of bacterial species including clinical isolates (Table 2). The peptide was effective against all the species. The peptide showed effectiveness at slightly lower concentrations for Gram positive bacteria (*S. aureus*, both methicillin sensitive and resistant, *S. epidermidis* and

enterococcus spp.) with MICs ranging between 0.08-0.52 mM when compared to Gram negative bacteria (*P. aeruginosa*, *K. pneumoniae*, *E. coli* and *E. cloacae*) which had MICs between 0.67 – 0.95 mM).

AMP were tested against bacteria commonly found in implant infections (*S. aureus* and *S. epidermidis*) to try to ascertain the AMP ability to generate resistant bacteria. When the bacteria were preconditioned in two different ways to the peptide to generate resistance, no resistance was observed. However, the pre-treatment of the bacteria with peptide did affect the MIC value for the peptide in each strain. Incubation overnight of the bacteria in half the MIC of the peptide did reduce the MIC concentration in *S. epidermidis* (Table 3). Consecutive incubation of the bacteria with increasing concentration of peptide resulted in the MIC decreasing at each concentration for both *S. aureus* and *S. epidermidis* (Figure 3).

Antimicrobial efficacy AMP coating against clinical isolates

eAMP, cAMP and dAMP coated and uncoated HA were tested to determine the efficacy of the surface against clinical isolates commonly found in orthopaedic infections. The surfaces were trialled against *S. aureus*, *S. epidermidis* and *P. aeruginosa* the most commonly found bacteria in orthopaedic infections (Figure 4). The data demonstrated that both the cAMP and dAMP could inhibit growth of bacteria on the hydroxyapatite surfaces (Figure 4a) while eAMP coating alone could not significantly reduce bacterial colonisation of the surface.

Swabs of both cAMP and dAMP surfaces showed no colony forming units against any of the bacteria tested (Figure 4a). However, the cAMP coating could only reduce the levels of planktonic bacteria in solution around the surface. While the eAMP and dAMP coatings could successfully inhibit planktonic growth of the bacteria in solution and bacterial colonisation on the surface (Figure 4b).

Efficacy of the coating after incubation in serum

Following incubation in serum the efficacy of the dAMP at inhibiting growth of bacteria in solution was not significantly different from dAMP coated HA which had not been exposed to serum. Both surfaces were significantly lower than uncoated HA ($p < 0.001$) (Figure 5a). However, following incubation in serum the efficacy of inhibiting bacteria on the surface was slightly although not significantly reduced when compared to dAMP coated HA which had not been exposed to serum (Figure 5b). Both dAMP coated HA and dAMP coated HA which had been incubated in serum had significantly lower bacterial colonisation than uncoated HA ($p < 0.001$).

Long term antimicrobial efficacy against *S. epidermidis*

dAMP-tHA pellets incubated with *S. epidermidis* over 3 weeks. The optical density of the solution surrounding the pellets was measured each week which showed that HA surfaces had significantly higher bacteria number with optical densities of 0.12 ± 0.02 at week 1, increasing to 0.27 ± 0.04 at week 2 and 0.35 ± 0.06 at week 3. Compared to dAMP-tHA surfaces at week 1 (0.02 ± 0.01), week 2 (0.05 ± 0.02) and week 3 (0.09 ± 0.02) (Figure 6a). After 3 weeks the surfaces were removed from the LB Broth and swabs of the surfaces were plated to show that the dAMP-tHA surfaces still had not been colonised by the bacteria in this time compared to HA surfaces which showed high bacterial levels ($5.8 \times 10^4 \pm 5.3 \times 10^4$) (Figure 6b).

Toxicity of the peptide in culture with mammalian cells.

The toxicity of the peptide alone was tested in both osteoblast precursor cells and osteosarcoma cells (Figure 7a-b). The peptide demonstrated no toxicity in either cell type over a 7 day period. In osteoblast precursor cells the cell number increases steadily over 1 week from $1.7 \times 10^5 \pm 2.5 \times 10^5$ (control) and $2.0 \times 10^5 \pm 1.5 \times 10^5$ (peptide) at day 1 to $1.7 \times 10^6 \pm 1.7 \times 10^5$ (control) and $1.7 \times 10^6 \pm 1.2 \times 10^5$ (peptide) as the cells reach confluency at day 7. In osteosarcoma cells the cell number increases from $9,895 \pm 96$ (control) and $9,391 \pm 113$ (peptide) at day 1 to day 3 $29,880 \pm 598$ (control) and $23,149 \pm 620$ (peptide). The cell number plateaus over the following four days with final readings of $23,119 \pm 495$ (control) and $24,718 \pm 2,166$ (peptide). Both growth curves match the control samples of cells grown without peptide present in the cell media with no statistical differences between the cell number with or without peptide (Figure 7).

Toxicity of the dAMP coating on HA to mammalian cells.

Osteoblast precursor cells were cultured on dAMP-tHA and HA for 14 days (Figure 8 a-b). In both treated and untreated HA the cell number increased up to day 7, $1.6 \times 10^6 \pm 1.8 \times 10^5$ (peptide coated) and $1.6 \times 10^6 \pm 2.2 \times 10^5$ and then plateaued. Although the cell number appears to decrease at day 14 there was no significant difference between day 9 peptide coated ($1.4 \times 10^6 \pm 1.5 \times 10^5$), control ($1.5 \times 10^6 \pm 7.9 \times 10^5$) and day 14 peptide coated ($1.1 \times 10^6 \pm 6.2 \times 10^5$), control ($1.0 \times 10^6 \pm 4.6 \times 10^5$). The cell number followed the same growth pattern on both dAMP-tHA and HA with no significant differences between the two groups. Over the 14 days the osteosarcoma cell number increased up to day 3 peptide coated ($2.2 \times 10^4 \pm 395$), control ($2.9 \times 10^4 \pm 1713$) and then plateaued with no significant differences between the time points day 3-14, peptide coated ($2.3 \times 10^4 \pm 158$), control ($2.6 \times 10^4 \pm 1133$) (Figure 8).

There were no significant differences between the dAMP-tHA and HA groups indicating that the dAMP did not demonstrate toxicity to the cells when bound onto a surface.

Discussion

As infection is established as a major complication of orthopaedic surgery, there have been multiple attempts to manipulate the host-device interface by the addition of antimicrobial agents^{17,18}. While there has been a good deal of success, the main barrier to clinical translation has been the lack of ability to tailor implant material chemistry at a molecular scale and a reliance on incorporating agents with a secondary material phase on to the implant surface. An implant formed from titanium with a polymer surface coating, which is then covalently functionalised changes the properties of the implant for the host tissue and correspondingly the host tissue response to the device.

By binding peptides to the surfaces of material such as hydroxyapatite directly we aim to leave as much of the original material 'visible' to the host tissue as possible and therefore retain the osseointegrative properties of the implant the peptide coating has a surface density of 1 molecule / nm. This suggesting the cells will still be able to attach to the hydroxyapatite. While previous studies have demonstrated that the idea of dual coating is possible on different surfaces, all of the coatings rely on the deposition of polymers which mask the surface completely changing the material surface properties exposed to the *in vivo* environment to the body⁴⁶. In this work we are tailoring the surface chemistry of the implant and tethering the antimicrobial peptides directly onto the surface in order to inhibit bacterial growth. The scanning electron microscopy images support that the macroscale of the material is unchanged and the cell growth experiments have shown that the nanoscale changes have not affected the cell response to the material.

The defensin sequence synthesised, based on human defensin sequences, showed comparable levels of antimicrobial efficacy to human defensins⁴⁷. While these values can vary depending on the environment, this comparison crucially demonstrates that the efficiency of the antimicrobial sequences is unchanged by the shorter peptide length and simplification of the sequence. One real potential of the peptide sequence presented here is the apparent inability of the bacteria to develop resistance in the studies carried out. This is believed to be due to the proposed mechanism of action of the peptide. Antimicrobial peptides are known to insert into bacterial cells causing pore formation⁴⁸. This destabilises the membrane and triggers cell lysis and death⁴⁹. The positively charged AMP will associate strongly with the negative charges found in the bacterial cell wall⁵⁰. This allows the hydrophobic regions of the peptide to induce pore formation. The bacteria will struggle to develop resistance to this as it would demand either removal or complete restructuring of the bacterial cell wall. With antimicrobial resistance becoming a global problem, developing antimicrobial surfaces for clinical applications, against which bacteria struggle to present resistance to is of great interest to the clinical community⁵¹. While further studies are of course required, this presents a promising outlook for the AMP. Long term studies into antimicrobial coated implants have demonstrated that after 5 years the implants are colonised and the bacteria are resistant to the chosen antibiotic⁵². By using a broad spectrum antimicrobial peptide (that the bacteria cannot rapidly develop resistance to) ensures that colonisation of the surface will not occur.

Mikos *et al* demonstrated that short sequence polyglutamic acid residues can bind strongly to the surface of hydroxyapatite⁵³. Our work here has demonstrated that the inclusion of

the binder sequence does not hinder the efficacy of the peptide and ensures that the solution around the surface can be sterilised in the short term. In the long term studies the efficacy of the surface at sterilising the solution surrounding it was reduced as the electrostatically bound peptide was removed over sequential broth changes.

The stability of the cAMP to long term incubation supports the spectroscopic data that the peptide has formed a covalent bond with the surface which will not allow the peptide to desorb from the surface. The apparent stability of the peptides to proteolytic degradation is due to steric hindrance. As the peptides are grafted directly to the surface without linker spacer units, the hydroxyapatite surface provides a large amount of steric hindrance around the peptides. The high coverage of peptide across the surface also provides steric bulk preventing access to the peptide chain. This prevents the enzymes such as proteases reaching the regions on the chain it can activate, and gives the AMP coating its high stability profile.

The stability of the covalent functionality of the dAMP coating also supports translation of this work. The coating is shown to be stable up to 12 months in simulated body fluid with motion and change of the solution surrounding the surface. This suggests that the coating will retain stability in the body and give a long lasting coating on the implant preventing biofilm formation without displaying cytotoxicity. The long term microbiological efficacy study demonstrated that not only did the surface maintain its efficacy in optimum bacterial growth conditions, but that the dAMP surface still significantly reduced the number of planktonic bacteria in solution despite the eAMP coating dissociation and removal in the initial broth changes. This demonstrates that the covalent bond form of the peptide is

effective at maintaining surface sterilisation and may reduce bacteria number overall in a closed system by eliminating bacteria when they come into contact with the surface.

Previous work on the surface chemistry of hydroxyapatite by Williams *et al* has shown that it is possible to alter the surface chemistry of the hydroxyapatite to include thiol residues without significantly altering the material properties⁵⁴. This has allowed us to use the thiol groups to act as tethering sites to covalently link the antimicrobial peptides. This gives permanent functionalisation with the peptide without compromising the bulk properties of the material as evidenced by the SEM images. From a biological perspective cell proliferation and metabolic activity studies showed the AMP coating had no cytotoxic effects.

The results we present here have shown that the thiol modification has occurred (both EDX and Raman data, Table 1 and Figure s1). The EDX also demonstrates that we can develop this technique further and attach the antimicrobial peptides to the free thiol functionality. The fluorescence spectroscopy images demonstrates that this technique can be used to coat bulk material over large surface areas. This makes the case for translation of the dAMP coating into clinical application very strong.

The development of these dual surface coatings which impart both short term and long term antimicrobial functionality are vitally important for orthopaedic infection as infection can occur at the time of surgery or at later time. This results in a need for a dual coating which can sterilise the area surrounding the implant at the time of surgery and give long lasting functionality^{55,56}. The data presented here demonstrates that although both cAMP

and eAMP are efficacious, neither alone can inhibit both the planktonic and surface growth of bacteria. The dAMP coating can inhibit both.

In summary, in the fight to treat infection following implantation of a prosthesis, there are several important needs which must be met within the host-device interface: 1) Short term delivery of antimicrobial agents to sterilise the host tissue and device to prevent colonisation by bacteria. 2) Long term stable antimicrobial coatings to prevent bacterial colonisation of the implant surface. 3) No alteration of the material properties of the implant device which would compromise the ability of the implant to integrate with native bone tissue. To the best of our knowledge, these needs are unmet at the current state of the art in the field. There are many studies showing coating of orthopaedic devices by dip/spray coating or incorporation of antimicrobial agents during production and these give excellent materials^{57,58}. However, these materials only provide short term antimicrobial activity (<12 months), failing to tackle the whole problem. There has been further work on coating devices with polymers, which can then have antimicrobial agents 'permanently' attached but this method changes the properties of the implant material due to the inclusion of the polymer material. We have addressed this problem by incorporating the dual antimicrobial surface coating which facilitates short term release as well as a long term stable antimicrobial presence at the material surface.

Conclusions

We have demonstrated the manufacture of a novel dual system approach to prevent antimicrobial infections which utilises two different binding mechanisms for the same antimicrobial sequence. The electrostatically released peptide inhibits bacterial growth in

solution while the covalently bonded peptide inhibits adherence and biofilm formation. Both together can inhibit the colonisation of bacteria at the interface of an engineered surface with tissue, while maintaining cell growth. The peptides can be tailored to interact with a surface and maintain good efficacy against a broad spectrum of bacteria and show strong antimicrobial activity against clinical isolates of *S. aureus*, *S. epidermidis* and *P. aeruginosa* (the bacteria most commonly found in joint infections). By using our mix and match system combining multiple fragments with different properties (surface binding and antimicrobial activity) without compromising the individual functions, the dAMP coating gives short term sterilisation of the neighbouring tissue, followed by long-term sustained antimicrobial activity of the surface. With this work established, further work is needed to test the peptide coatings in a preclinical animal infection model to move them towards a clinical environment.

Acknowledgments

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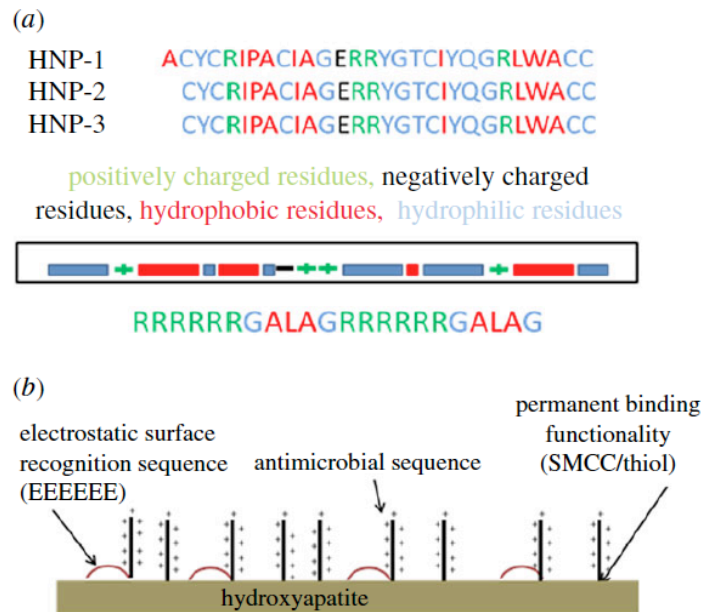


Figure 1. (a) The sequence of human defensins a1, 2 and 3 with the nature of the amino acids highlighted. The main patterns are then shown pictorially. The sequences of the first- and second-generation peptides are shown with the nature of the amino acids indicated by colour. (b) The attachment of the peptide to the HA through both electrostatic binding and covalent linkages.

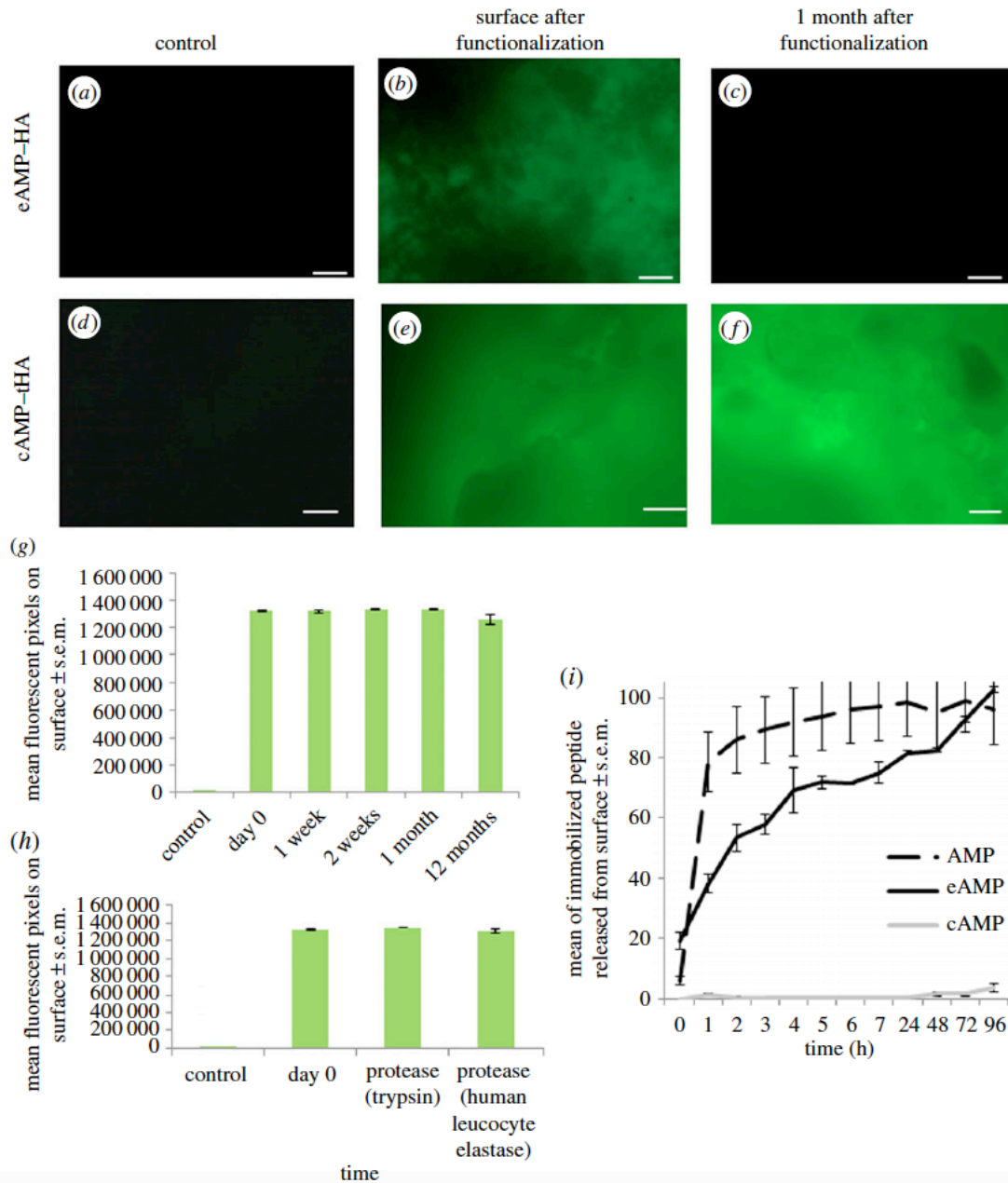


Figure 2. Fluorescence microscopy images of: (a) HA; (b) HA coated with electrostatic coating, imaged immediately after washing following coating; (c) HA coated with electrostatic coating, after samples had been stored in simulated body fluid for one month with changes every 7 days; (d) thiol-modified HA; (e) thiol-modified HA with covalent coating, imaged immediately after washing; (f) thiol-modified HA with covalent coating, after samples had been stored in simulated body fluid for one month with changes every 7 days before. All images are representative of all sample surfaces. (g) Quantification of the fluorescence on the covalently coated, thiol-modified HA over time, showing the average surface pixel count, $n = 3$, and error bars show the standard error of the mean. (h) Quantification of the fluorescence on the covalently coated, thiol-modified HA after treatment with proteases, $n = 3$, and error bars show the standard error of the mean. (i) Release from the eAMP, cAMP and AMP coatings from the surface over 96 h, $n = 3$, error bars show the standard error of the mean.

Table 1: Table 1. Elemental composition (% wt) of the substrate and functionalized substrate as determined through EDX measurements. Values shown are the averages \pm standard deviation of 5 readings taken at different points across the surface of each pellet.

	Ca	P	C	O	Si	S
Hydroxyapatite	29.39 \pm 0.64	16.74 \pm 0.74	6.93 \pm 0.35	46.94 \pm 0.44	0 \pm 0	0 \pm 0
Hydroxyapatite – thiol modified	31.62 \pm 0.93	16.25 \pm 0.43	8.78 \pm 0.44	42.40 \pm 1.13	0.20 \pm 0.08	0.37 \pm 0.10
Hydroxyapatite dAMP	19.50 \pm 2.72	11.94 \pm 1.28	25.95 \pm 4.76	41.99 \pm 1.76	0.24 \pm 0.04	0.39 \pm 0.04

Table 2: Minimum Inhibitory Concentration (MIC) of AMP against clinically isolated bacteria.

Bacteria	MIC (mM) \pm SEM
<i>P. aeruginosa</i>	0.87 \pm 0.19
<i>K. pneumoniae</i>	0.95 \pm 0.13
<i>E. Coli</i>	0.67 \pm 0.18
<i>Enterobacter cloacae</i>	0.74 \pm 0.18
<i>S. aureus (methicillin sensitive)</i>	0.37 \pm 0.03
<i>S. aureus (methicillin resistant)</i>	0.35 \pm 0.07
<i>S. epidermis (Coagulase negative)</i>	0.08 \pm 0.03
<i>Enterococcus spp.</i>	0.52 \pm 0.31

Table 3: The effect of peptide pre-incubation with bacteria on Minimum Inhibitory Concentration

Bacteria	MIC (naïve bacteria)	MIC (preconditioned 24 hours)
S. aureus (NCTC 8532)	0.9 mM	0.9 mM
S. Epidermis (C8 - clinical isolate)	0.9 mM	0.2 mM

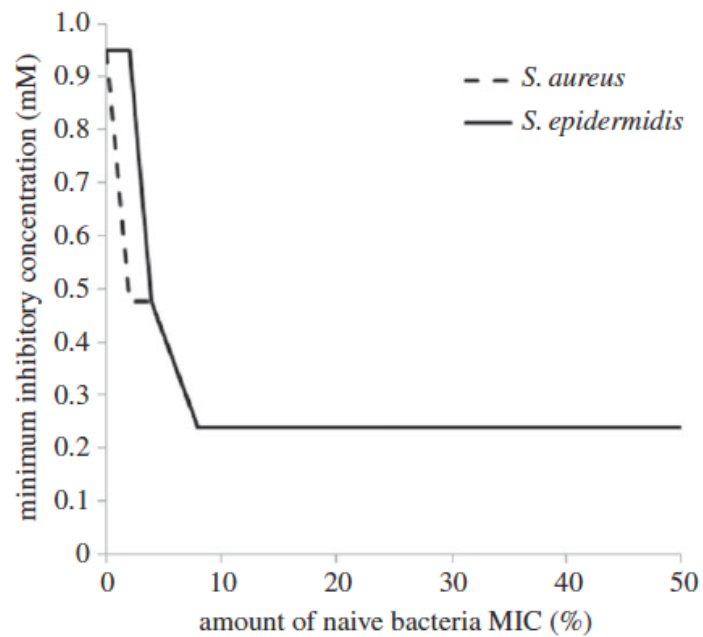


Figure 3. MIC for *S. aureus* and *S. epidermidis* after incubation in increasing concentrations of AMP, shown as a percentage of the initial MIC required to inhibit growth in naive bacteria.

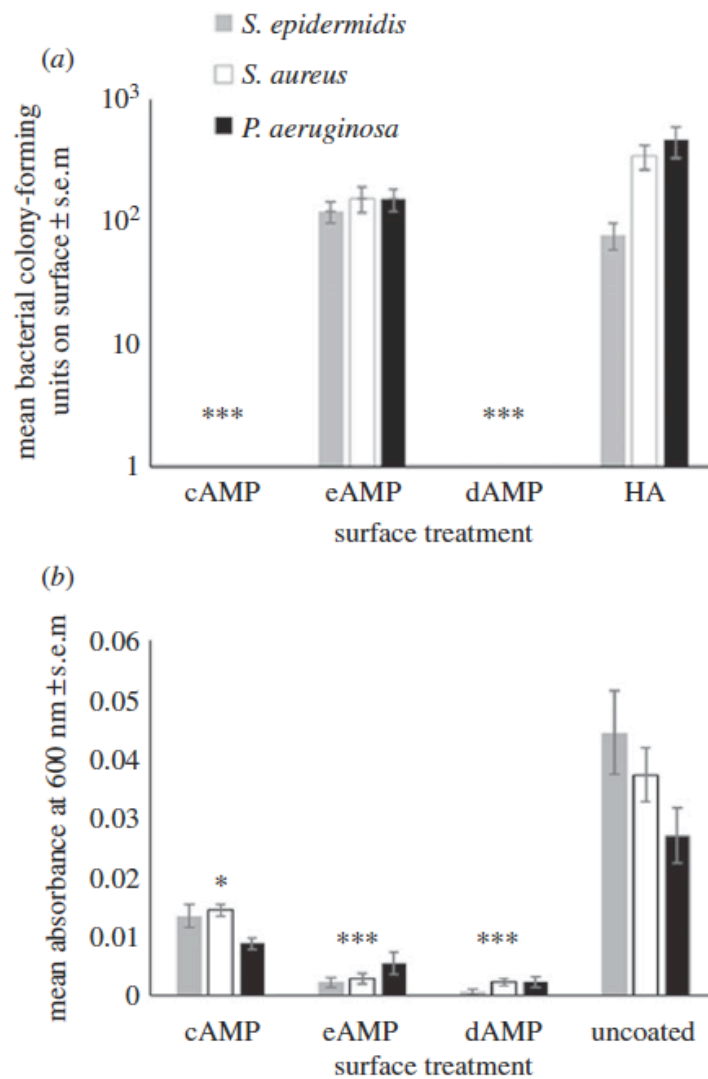


Figure 4. (a) The number of colony-forming units found on cAMP–tHA, dAMP–tHA or tHA after incubation with *S. aureus*, *S. epidermidis* and *P. aeruginosa*. Error bars show standard error of the mean, $n = 6$, *** $p < 0.000$. (b) The absorbance of the bacteria in broth surrounding the surface at 600 nm for *S. epidermidis*, *S. aureus* and *P. aeruginosa* in the solution surrounding a cAMP–tHA/eAMP–tHA/dAMP–tHA or tHA. Error bars show standard error of the mean, $n = 6$, ** $p < 0.001$, * $p < 0.05$.

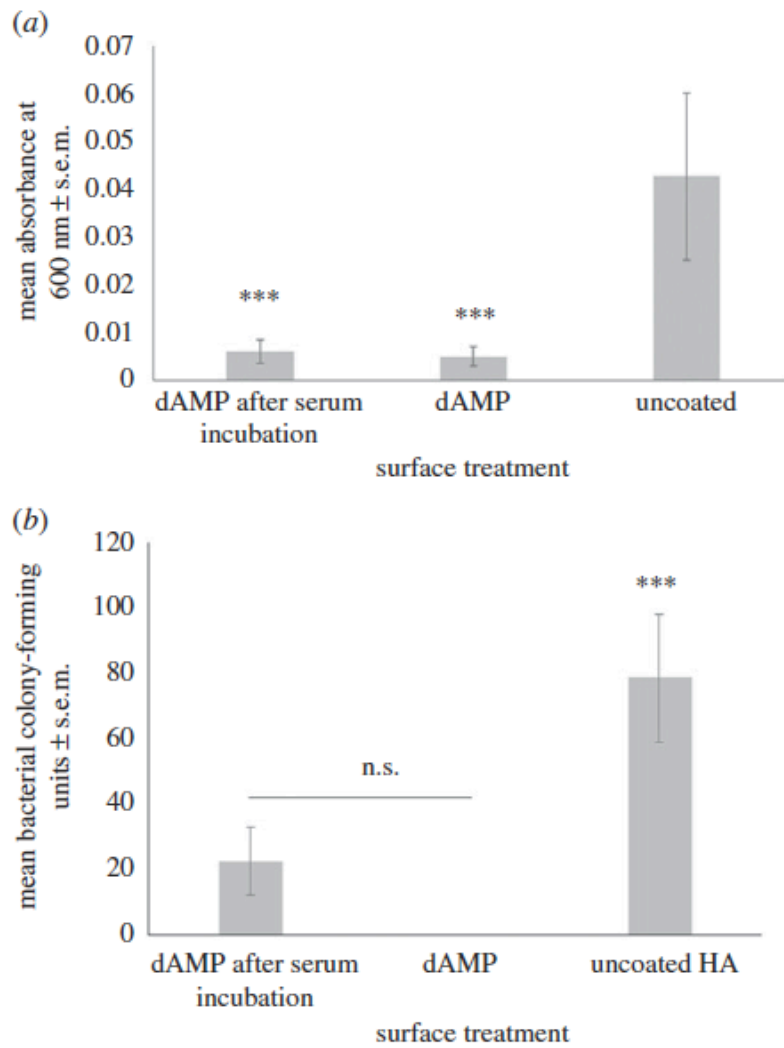


Figure 5. (a) The number of colony-forming units of *S. aureus* found on dAMP-tHA or tHA with and without a 4 h incubation in serum. Error bars show standard error of the mean, $n = 6$, *** $p < 0.000$. (b) The absorbance of the bacteria in broth surrounding the surface at 600 nm of dAMP-tHA or tHA with and without a 4 h incubation in serum. Error bars show standard error of the mean, $n = 6$, *** $p < 0.001$, * $p < 0.05$.

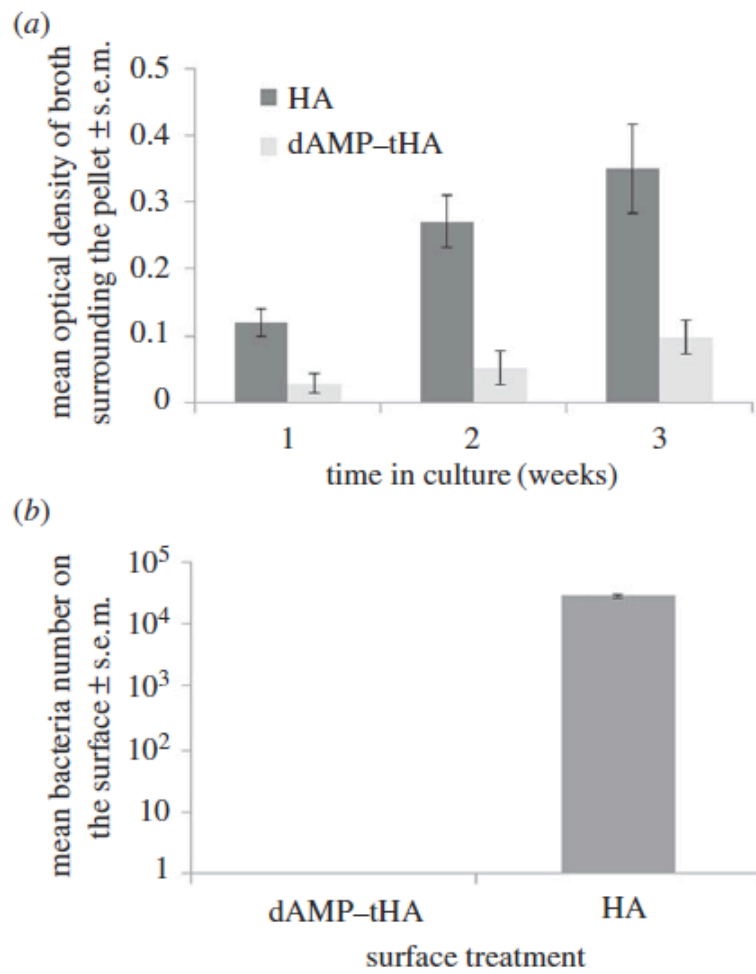


Figure 6. (a) The survival of *S. epidermidis* in the solution surrounding the dAMP-tHA pellet at 1, 2 and 3 weeks compared with HA measured using optical density. (b) Survival of *S. epidermidis* on the surface of the dAMP-tHA pellet compared with the HA pellet after three weeks in culture. Error bars show standard error of the mean, $n = 6$.

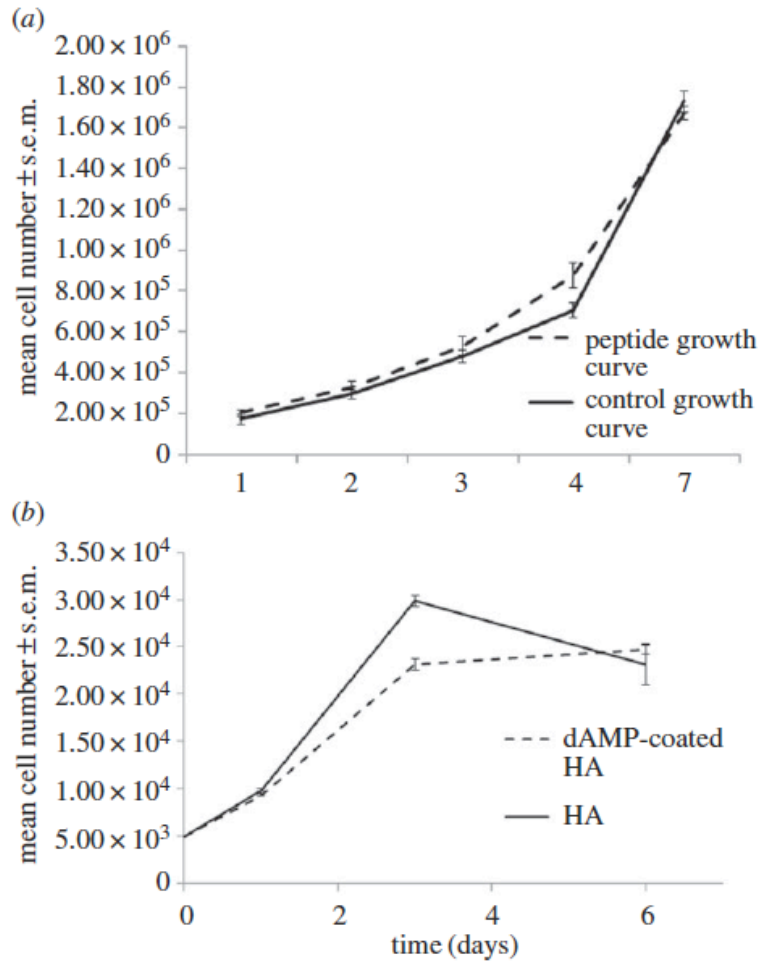


Figure 7. (a) Osteoblast precursor cells grown on tissue culture plastic with and without AMP in the cell media throughout growth. (b) Osteosarcoma cells grown on tissue culture plastic with and without AMP in the cell media, $n = 6$, error bars show standard error of the mean.

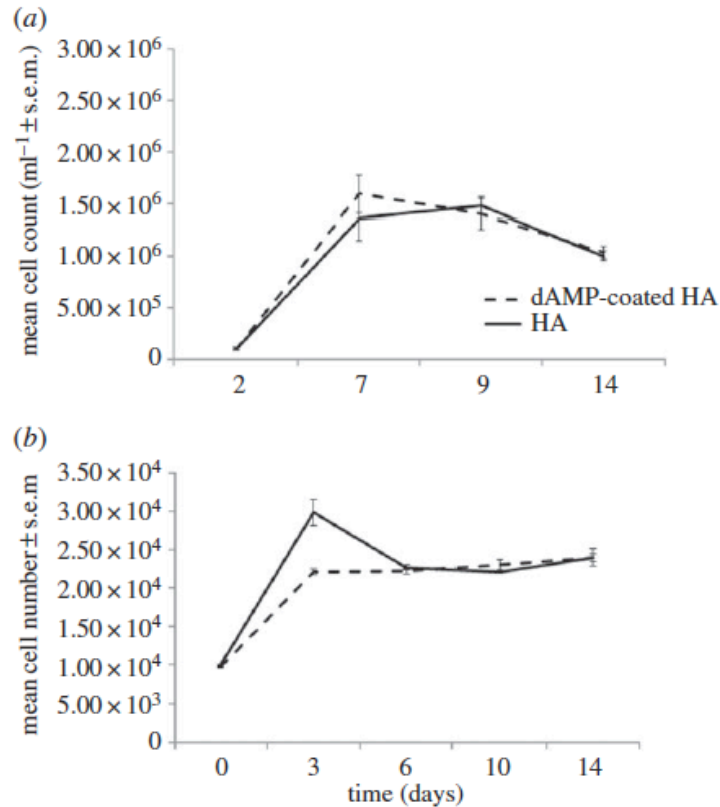


Figure 8. (a) Osteoblast precursor cells grown on dAMP–tHA pellets compared with osteoblast precursor cells grown on HA pellets. (b) Osteosarcoma cells grown on dAMP–tHA pellets compared with HA pellets. Error bars show standard error of the mean, $n = 6$.

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